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Effect of Blood Hematocrit and Erythrocyte Deformability on Adenosine 5′-Diphosphate Platelet Reactivity in Patients With Acute Coronary Syndromes on Dual Antiplatelet Therapy

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Previous studies have explored the association between hemorheologic alterations and aspirin resistance, pointing out the possible interaction between hematologic components and platelet responsiveness to antiplatelet drugs. The aim of this study was to evaluate the association between hemorheologic variables and residual platelet reactivity in patients with acute coronary syndromes (ACSs) who underwent percutaneous coronary intervention on dual antiplatelet therapy. The study population included 528 patients with ACSs. Hemorheologic studies were performed by assessing whole blood viscosity at 0.512 and 94.5/second, plasma viscosity, and erythrocyte deformability index. Post-treatment platelet reactivity was investigated by measuring platelet aggregation by adenosine 5′-diphosphate (ADP) 10 μmol and a value >70% was defined as high ADP platelet reactivity. Significantly ($p < 0.01$) lower values of hematocrit and erythrocyte deformability and higher values of whole blood viscosity at 94.5/second were found in patients with high ADP platelet reactivity. At multivariate analysis, lower values of hematocrit and erythrocyte deformability index and higher values of whole blood viscosity at 94.5/second and leukocytes (highest vs lowest tertile) also resulted in an independent association with high platelet reactivity, except for leukocytes, after simultaneous adjustment for hematocrit, leukocyte count, and erythrocyte deformability index. In conclusion, these results demonstrate the influence of hematocrit and of erythrocyte deformability on ADP platelet reactivity. These variables could be considered to optimize treatment with antiplatelet therapy in these patients. **(Am J Cardiol 2009;104:764–768)**

Use of clopidogrel in addition to acetylsalicylic acid has been demonstrated to be associated with a decrease of cardiovascular events in patients with acute coronary syndromes (ACSs) independently of stent implantation.[1,2] Retrospective studies have shown that discontinuation of clopidogrel, even ≥6 months after stent implantation, is associated with an increased risk of thrombotic events in patients with drug-eluting stents.[3,4] However, interindividual variabilities in response to clopidogrel and acetylsalicylic acid have been reported in patients with ACS.[5] From these assumptions, the phenomenon of clopidogrel resistance has progressively gained great interest[6,7] and recent studies have shown that decreased responsiveness to clopidogrel is associated with an increased incidence of death and cardiovascular events[8] and is a strong predictor of stent thrombosis after drug-eluting stent implantation.[9] Previous studies have reported an association between hemorheologic variables and aspirin resistance.[10,11] The aim of this study was to evaluate the possible association between hemorheologic variables and residual platelet reactivity to adenosine 5′-diphosphate (ADP) in patients with ACS on dual antiplatelet therapy.

Methods

The study population included 528 consecutive patients with a diagnosis of ACS, 272 with ST-segment elevation myocardial infarction (STEMI), and 256 with non-STEMI or unstable angina pectoris (UAP) admitted from March 2004 to December 2006 to the intensive and postintensive coronary care units of the department of medical and surgical critical area of the Azienda Ospedaliero-Universitaria Careggi (Florence, Italy) after undergoing percutaneous coronary intervention (PCI).

Acute MI was diagnosed from an increase in creatine kinase-MB isoenzyme ≥2 times the upper limit (3.6 ng/ml) and/or increased cardiac troponin I (>0.15 ng/ml) levels with ≥1 of the following: acute onset of prolonged (≥20 minutes) typical chest pain, ST-segment elevation of ≥1 mm in ≥2 contiguous electrocardiographic leads, or new left bundle branch block on 12-lead electrocardiogram for a diagnosis of STEMI, and ST-segment depression of ≥0.5 mm 0.08 second after the J point in ≥2 contiguous leads or T-wave inversion >1 mm in leads with predominant R

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Table 1
Clinical characteristics of subjects investigated

Variable	Low Reactivity (n = 427)	High Reactivity (n = 101)	p Value
Age (years), median (range)	68 (28–92)	73 (22–94)	0.001
Men/women	266/161	63/38	1.0
Hypertension*	250 (58.5%)	64 (63.4%)	0.4
Dyslipidemia†	173 (40.5%)	40 (39.6%)	0.9
Smoker	185 (43.3%)	43 (42.6%)	0.9
Diabetes mellitus‡	86 (20.1%)	26 (25.7%)	0.2
Obesity§	83 (19.4%)	22 (21.8%)	0.6
Family history of coronary artery disease‖	136 (31.9%)	42 (41.6%)	0.08
History of coronary artery disease¶	162 (37.9%)	44 (43.6%)	0.3
Previous PCI	95 (22.2%)	23 (22.8%)	0.9
Previous coronary artery bypass grafting	27 (6.3%)	4 (4.0%)	0.5
STEMI	216 (50.6%)	56 (55.4%)	0.4
Non-STEMI	76 (17.8%)	16 (15.8%)	0.8
UAP	135 (31.6%)	29 (28.7%)	0.6
Multivessel disease	300 (70.3%)	63 (62.4%)	0.2
Culprit coronary artery			
Left main	20 (4.7%)	5 (5.0%)	1.0
Left anterior descending	203 (47.5%)	50 (49.5%)	0.7
Right	123 (28.8%)	32 (31.7%)	0.6
Left circumflex	75 (17.6%)	14 (13.9%)	0.5
Others	9 (2.1%)	0 (0.0%)	0.2
Renal dysfunction#	26 (6.1%)	8 (7.9%)	0.5
Bleeding complications	38 (0.09%)	12 (0.12%)	0.3
Angiotensin-converting enzyme inhibitors	387 (90.6%)	90 (89.1%)	0.7
β blockers	367 (85.9%)	77 (76.2%)	0.02
Statins	385 (90.2%)	86 (85.1%)	0.2
Diuretics	236 (55.3%)	55 (54.5%)	0.9
Nitrates	257 (60.2%)	68 (67.3%)	0.2
Glycoproteins IIb/IIIa inhibitors	221 (51.8%)	36 (35.6%)	0.004

* Defined as the presence of blood pressure values >140/90 mm Hg according to guidelines of the European Society of Hypertension/European Society of Cardiology and/or antihypertensive treatment.

† Defined according to the Third Report of the National Cholesterol Education Program.

‡ Defined according to the American Diabetes Association.

§ Defined as a body mass index >30 kg/m^2.

‖ Defined as the presence of ≥1 first-degree relative who had developed coronary artery disease before age 55 years for men and 65 years for women.

¶ Defined as a history of MI or stable and unstable angina.

Defined as a creatinine clearance <60 ml/min/m^2.

waves for the diagnosis of non-STEMI. STEMI was diagnosed in 272 patients and non-STEMI in 92. UAP was defined according to Braunwald classification and only patients with class IIIb UAP were enrolled. All patients underwent coronary angiography performed by the Judkins technique and PCI; coronary vessels were defined as diseased when stenosis >70% was detected.

Traditional cardiovascular risk factors, assessed by patient interviews, echocardiography, and hospital records, were defined as presented in Table 1. Bleeding complications, major and minor, were defined according to Voeltz et al.[12] Exclusion criteria for patients with STEMI were a total latency period >12 hours and creatinine serum levels >4 mg/dl. Patients' demographic and clinical data were collected. All subjects gave their informed consent and the investigation was approved by the institutional committee on human research.

Blood samples for hemorheologic parameters and platelet reactivity assessment were collected simultaneously in the morning ≥6 hours after PCI but <48 hours after the occurrence of symptoms. A clopidogrel 600-mg loading dose was administered to each patient before PCI. For patients receiving in the catheter laboratory the loading dose of clopidogrel and a glycoprotein IIb/IIIa inhibitor, blood samples were obtained after ≥48 hours while the patient was on clopidogrel 75-mg maintenance dose. Hemorheologic studies were performed by assessing whole blood viscosity, plasma viscosity, and erythrocyte deformability index as previously reported.[13] Whole blood viscosity and plasma viscosity were measured at 37°C using the Rotational Viscosimeter LS 30 (Contraves, Zurich, Switzerland). Whole blood viscosity was analyzed at shear rates of 0.512 and 94.5/second. Plasma viscosity testing was performed at shear rate of 94.5/second. Erythrocyte filtration was measured by a microcomputer-assisted filtrometer (model MF4, Myrenne GmbH, Roetgen, Germany). For platelet reactivity assessment blood samples were obtained after clopidogrel loading and were anticoagulated with sodium citrate 0.129 mol/L (ratio 9:1). Platelet-rich plasma, obtained by centrifuging whole blood for 10 minutes at 200g, was stimulated with ADP 10 μmol/L (Mascia Brunelli, Milan, Italy) and residual aggregation was assessed using a APACT 4 light transmittance aggregometer (Helena Laboratories, Milan, Italy). The 100% line was set using platelet-poor plasma and 0 baseline established with platelet-rich plasma (adjusted from 18 up to 30 $\times$ 10^9/L). Platelet aggregation (according to the Born method) was evaluated by considering maximal percent platelet aggregation in response to a stimulus. According to data from the literature and our group,[8,9] patients with platelet aggregation by ADP 10 μmol ≥70% (90th percentile of controls) were considered as having high ADP platelet reactivity. For the different hemorheologic procedures, intra-assay coefficients of variation were <1.6% and interassay coefficients of variation were <4.5%. Fibrinogen was measured by clotting assay; intra- and interassay coefficients of variation were 2.2% and 3.8%, respectively. Mean coefficient of variation for ADP-induced platelet aggregation was 6.8%.

Statistical analysis was performed using SPSS 11.5 for Windows (SPSS, Inc., Chicago, Illinois). Data are given as median and range. To investigate the association between clinical and hemorheologic variables and residual platelet reactivity, patients with ACS were divided in 2 groups according to the presence of high residual platelet reactivity on dual antiplatelet therapy. Categorical variables were compared using chi-square test. Mann-Whitney test was performed to evaluate differences in hemorheologic variables in relation to the presence of high ADP platelet reactivity. Correlations between hemorheologic variables and platelet aggregation measured with ADP 10 μmol were performed by Pearson test.

Multinomial regression analyses were performed to evaluate the independent association between high ADP platelet reactivity and hemorheologic variables using residual plate-

Table 2
Hemorheologic parameters in relation to high adenosine 5′-diphosphate platelet reactivity

Variable	Low Reactivity (n = 427)	High Reactivity (n = 101)	p Value
Hematocrit (%)	37.9 (24.7–54.9)	36.4 (23.7–44.8)	0.002
Whole blood viscosity at 0.512/s (mPa/s)	24.16 (18.16–45.21)	24.93 (19.15–38.71)	0.6
Whole blood viscosity at 94.5/s (mPa/s)	4.38 (3.80–6.71)	4.40 (3.80–5.81)	0.001
Plasma viscosity (mPa/s)	1.48 (1.25–2.20)	1.50 (1.28–2.21)	0.1
Erythrocyte deformability index	5.35 (1.56–13.68)	2.00 (1.50–12.00)	0.000
Fibrinogen (mg/dl)	452 (223–1,720)	469 (158–890)	0.3
Leukocytes $\times 10^9$/L	8,290 (4,190–22,590)	9,140 (4,110–24,160)	0.2
Platelets $\times 10^9$/L	195 (100–573)	191 (101–617)	0.6

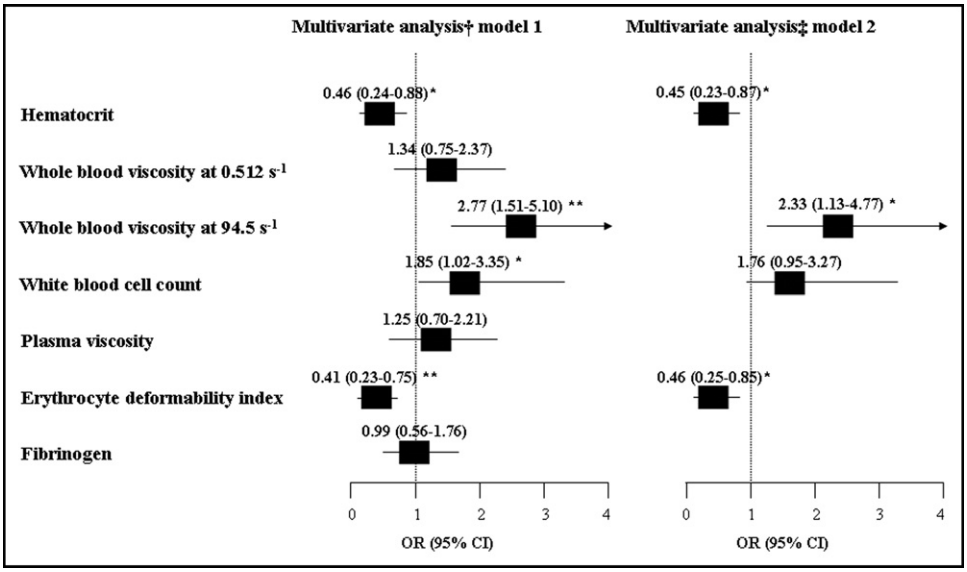

Figure 1. Multivariate logistic regression analysis for high (≥70%) ADP platelet reactivity in patients with ACSs. *p <0.05; **p <0.01. †Adjusted for gender, age, arterial hypertension, smoking habit, dyslipidemia, diabetes mellitus, obesity, family and personal histories of coronary artery disease, renal dysfunction, bleeding complications, and pharmacologic therapy. ‡Adjusted also for leukocyte count, hematocrit, and erythrocyte deformability. CI = confidence interval; OR = odds ratio.

let reactivity as a dependent variable. Variables generally accepted as influencing high ADP platelet reactivity were included in the analysis (age, gender, hypertension, dyslipidemia, smoking habits, diabetes mellitus, obesity, renal dysfunction, bleeding complications, pharmacologic therapy, family and personal histories of coronary artery disease) in a model (model 1) in which each hemorheologic variable was added separately. Another model in which results of hemorheologic variables, independently associated to high ADP platelet reactivity in model 1 analysis, were adjusted for white blood cell count, hematocrit, and erythrocyte deformability index was also performed (model 2).

All regression coefficients are given with 95% confidence intervals. A p value <0.05 was considered statistically significant.

Results

Clinical characteristics of patients are listed in Table 1. Bare metal stents were implanted in 147 of 449 patients with ACS and drug-eluting stents in 302 of 449. One hundred one of 528 patients with ACS showed persistently high ADP platelet reactivity on dual antiplatelet therapy administration.

Significantly lower values of hematocrit and erythrocyte deformability index and slightly higher values of whole blood viscosity at 94.5/second were found in relation to high residual platelet reactivity (Table 2). No significant difference was found between patients with high ADP platelet reactivity and those without in relation to type of stent implanted (bare metal or drug eluting). No significant difference in platelet count was found between patients with hematocrit values below the first tertile and the others.

Mild, but significant, correlations between platelet aggregation by ADP 10 μmol and hematocrit ($r = -0.26$, $p = 0.000$), erythrocyte deformability index ($r = -0.36$, $p = 0.000$), whole blood viscosity at 94.5/second ($r = 0.17$, $p = 0.000$), plasma viscosity ($r = 0.16$, $p = 0.000$), and fibrinogen ($r = 0.15$, $p = 0.000$) were observed.

At multinomial regression analyses hematocrit, erythrocyte deformability index, whole blood viscosity at 94.5/second, and leukocytes in the highest versus lowest tertile

and age, family history of coronary artery disease, and more limited use of glycoprotein IIb/IIIa inhibitors and β blockers resulted in an independent association with high ADP platelet reactivity in model 1 analysis (Figure 1). In model 2 analysis erythrocyte deformability index, hematocrit, and whole blood viscosity at 94.5/second in the highest versus lowest tertile remained independently associated with high ADP platelet reactivity (Figure 1).

Discussion

Previous studies have shown a significant association between a decreased responsiveness to clopidogrel and an increased rate of thrombotic events[8,9] and possible alternative therapeutic strategies have been suggested. In fact, it has been proposed to double the mean daily dosage of clopidogrel, which has been demonstrated to determine a more intense inhibition of platelet aggregation than the currently recommended 75-mg maintenance dose[14] or to administer a different antiplatelet drug such as ticlopidine or prasugrel, a third-generation thienopyridine that has been recently demonstrated to provide greater and faster P2Y(12) receptor-mediated platelet inhibition than clopidogrel.[15]

Moreover, a high clopidogrel loading dose in patients undergoing coronary stenting has been shown to optimize platelet inhibitory effects soon after intervention, possibly providing a more effective protection against early thrombotic complications.[16]

A possible role of hemorheologic variables in influencing aspirin resistance has been recently described,[10,11] confirming the role of erythrocytes and leukocytes in the pathogenesis of arterial thrombosis.

The novel finding of the present study is the significant association between some hemorheologic variables and high residual ADP platelet reactivity in patients with ACS on dual antiplatelet therapy. In particular, we found an independent association between decreased erythrocyte deformability and increased platelet aggregation induced by ADP. A possible explanation for this relation is that erythrocytes with decreased deformability tend to release a larger amount of ADP in the circulation compared to those without[17] and ADP released can compete against clopidogrel for purinergic receptors present on the platelet surface, thus explaining the high ADP platelet reactivity in these patients.

Thus, another possible target to increase clopidogrel responsiveness should be administration of drugs capable of improving erythrocyte deformability, among which one should consider administration of ω-3 fatty acids[18]; in fact, fish oil administration has been previously reported to determine a decrease in ADP release from shear-stressed erythrocytes by increasing their filterability.[17] Moreover, ω-3 fatty acids have been previously reported to determine a decrease in platelet aggregation,[19] a mechanism that could contribute, at least in part, to longer survival.

Another interesting finding of the present study is the mild but significant negative correlation between hematocrit and high ADP platelet reactivity. Because anemia has been previously reported to be associated with a higher rate of death and thrombotic events in patients with ACS,[20] the decreased responsiveness to clopidogrel found in our study in patients with ACS and lower hematocrit values could, at least in part, account for the higher rate of thrombotic events in these patients.

Moreover, this result agrees with those of a recent report showing an independent association between lower hematocrit values and aspirin resistance in patients with stable coronary artery disease.[21]

Decreased erythrocyte mass could be associated with a lower availability of nitric oxide, which has been recently demonstrated to be reversibly bound, transported, and released by erythrocytes in the cardiovascular system under physiologic and pathologic conditions.[22] Erythrocytes express an active and functional endothelium-type nitric oxide synthase, which is localized in the plasma membrane and the cytoplasm of erythrocytes[22]; in human erythrocytes nitric oxide regulates deformability of the erythrocyte membrane and inhibits platelet function due to an increase of intracellular cyclic guanosine monophosphate levels in platelets.[23] Moreover, it has been demonstrated that erythrocytes are capable of releasing adenosine triphosphate by mechanical deformation or pharmacologically, thus favoring nitric oxide release by activated platelets.[24]

In contrast, anemic patients with ACSs, except those with very low hematocrit, do not seem to benefit from administration of blood transfusions that, on the contrary, are associated with a worse outcome[25]; the negative impact of blood transfusions in patients presenting with anemia and suspected ACS has been confirmed and ascribed to the change in shape and deformability of stored erythrocytes,[25] which probably release a larger amount of ADP, thus further decreasing platelet responsiveness to antiplatelet drugs.

The association between anemia and higher values of platelet aggregation found in our patients could be explained by the presence of a large number of reticulated platelets.

Recently, a significant negative correlation has been reported between hematocrit and reticulated platelet number, which in turn has been associated with persistent platelet reactivity in patients on dual antiplatelet therapy.[26] Reticulated platelets are metabolically and enzymatically more active than smaller platelets; their increased number in anemic patients should be explained by a compensatory production from stimulated bone marrow of larger reticulated platelets.[27]

These patients, however, could benefit from administration of nitric oxide donors such as LA419, which has been recently reported to have antithrombotic properties.[28]

In the present study, leukocytes in the highest versus lowest tertile resulted in an independent association with high ADP residual platelet reactivity in model 1 analysis but not after adjustment for hematocrit levels and erythrocyte deformability index values. This result suggests that alterations of these latter variables play a pivotal role in influencing platelet responsiveness to antiplatelet drugs whose mechanism of action is mediated by ADP receptors.

A limitation of this study is that we did not provide the number of reticulocytes, which have been demonstrated to be responsible for decreased erythrocyte deformability in anemic patients with reticulocytosis.[29] Reticulocytes have lower deformability and lower membrane fluidity than mature red blood cells and these indexes normalize during maturation.[30] However, decreased erythrocyte deformabil-

ity and lower hematocrit values resulted in an independent association with high platelet reactivity, suggesting their independent role in modulating responsiveness to antiplatelet drugs.

Another limitation of the present study is that we did not measure ADP plasma levels in the 2 groups of patients with decreased and normal erythrocyte deformabilities, so we can only speculate that increased levels of ADP in patients with decreased erythrocyte deformability may explain the increased ADP platelet reactivity.

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